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## Targeting proteostasis in atrial fibrillation

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## Chapter 3

### Effects of different small HSPB members on contractile dysfunction and structural changes in a *Drosophila melanogaster* model for Atrial Fibrillation

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## ABSTRACT

**Background:** The most common clinical tachycardia, Atrial Fibrillation (AF), is a progressive disease, caused by cardiomyocyte remodeling, which finally results in contractile dysfunction and AF persistence. Recently, we identified a protective role of heat shock proteins (HSPs), especially the small HSPB1 member, against tachycardia remodeling in experimental AF models. Our understanding of tachycardia remodeling and anti-remodeling drugs is currently hampered by the lack of suitable (genetic) manipulatable *in vivo* models for rapid screening of key targets in remodeling. We hypothesized that *Drosophila melanogaster* can be exploited to study tachycardia remodeling and protective effects of HSPs by drug treatments or by utilizing genetically manipulated small HSP-overexpressing strains.

**Methods and results:** Tachypacing of *Drosophila* pupae resulted in gradual and significant cardiomyocyte remodeling, demonstrated by reduced contraction rate, increase in arrhythmic episodes and reduction in heart wall shortening, compared to normal paced pupae. Heat shock, or pre-treatment with HSP-inducers GGA and BGP-15, resulted in endogenous HSP overexpression and protection against tachycardia remodeling. DmHSP23 overexpressing *Drosophila*s were protected against tachycardia remodeling, in contrast to overexpression of other small HSPs (DmHSP27, DmHSP67Bc, DmCG4461, DmCG7409, DmCG14207). (Ultra)structural evaluation of the tachypaced heart wall revealed loss of sarcomeres and mitochondrial damage which were absent in tachypaced DmHSP23 overexpressing *Drosophila*.

**Conclusion:** Tachypacing of *Drosophila* resulted in cardiomyocyte remodeling, which was prevented by general HSP-inducing treatments and overexpression of a single small HSP, DmHSP23. Thus, tachypaced *Drosophila melanogaster* can be used as an *in vivo* model system for rapid identification of novel targets to combat AF associated cardiomyocyte remodeling.

## 1. INTRODUCTION

Atrial Fibrillation (AF) is the most common tachycardia in the clinical setting and it affects patients' cardiovascular function in a progressive and sustained manner.<sup>1</sup> AF is characterized by specific changes in electrical, structural and contractile function of the atrial cardiomyocytes, commonly denoted as 'remodeling'. Tachycardia remodeling underlies contractile dysfunction and the progressive and intractable nature of AF. Hence, there is great interest in developing anti-remodeling therapies directed at the targets underlying remodeling.<sup>2</sup> However, our understanding of tachycardia remodeling that contributes to AF progression and the effects of anti-remodeling drugs is currently hampered by the lack of suitable genetically manipulatable *in vivo* models. There are some *in vitro* cardiomyocyte cell models, using isolated atrial cardiomyocytes or the HL-1 atrial cardiomyocyte cell line<sup>3,4</sup>, that can be used for such purposes and that yielded initial suggestions regarding the protective effects of heat shock proteins (HSPs).<sup>3,5,6</sup> Whereas some of the concepts generated in these *in vitro* models could be confirmed in experimental canine models for AF<sup>3,7</sup>, the precise translation to the *in vivo* situation is hindered due to limited possibilities for genetic manipulations in larger animal models that beside canine models<sup>8</sup>, include AF models in goat<sup>9</sup> and sheep.<sup>10</sup> Although these animal models have been very useful in obtaining knowledge about concepts of electrical remodeling and contractile dysfunction, they lack the flexibility of dissecting the underlying molecular mechanisms and employing genetic or compound screens. In addition, experimental execution is extensive and expensive. Therefore, we utilize *Drosophila melanogaster* as an *in vivo* model system for tachycardia remodeling, since it has been recognized that *Drosophila* contains powerful genetics and provide tools to manipulate gene expression in a highly precise spatial and temporal fashion, by the use of a UAS/GAL4 system.<sup>11,12</sup> Furthermore, 85% of the *Drosophila* genes have human homologues<sup>11,13,14</sup>, including several genes that have been associated with human cardiac diseases, including heart failure, arrhythmias and dilated cardiomyopathy.<sup>12,13,15-18</sup> Moreover *Drosophila* contains a pumping heart.<sup>11-14</sup> In the current study we report on the development of a tachycardia model in *Drosophila*. In addition, we show, consistent with our previous findings in *in vitro* cardiomyocytes<sup>3,5</sup> and *in vivo* canine models for AF<sup>3,7</sup>, that induction of HSPs protects against tachypacing-induced contractile dysfunction. Furthermore, by using both functional and (ultra)structural analyses, it was found that this protection is accomplished upon overexpression of a single HSP, DmHSP23, the possible ortholog of human HSPB1. Moreover, overexpression of the other small DmHSP members did not result in a protective effect. Thus our study demonstrates the feasibility and power of the tachypaced *Drosophila melanogaster* as a model in translational research in the field of tachycardia.

## 2. MATERIALS AND METHODS

### 2.1. Maintenance of *Drosophila melanogaster* strains and HSP-inducing treatments

*Drosophila* stocks were maintained at 21°C, during experiments at 25°C, according to standard protocols. The W1118 line was used as a control and obtained from Genetic Services Inc. (Massachusetts, USA). Actin-GAL4 (stock #4414) driver strains were obtained from the Bloomington Stock Centre (Indiana University, USA). UAS-DmHSP23 and UAS-DmHSP27 strains were generated from W1118 genetic background and have been described before<sup>19-21</sup>. These UAS strains were crossed, using standard genetics, with the actin-GAL4 expressing strains in order to generate strains that contain actin-GAL4 and either UAS-DmHSP23 or UAS-DmHSP27 on the same chromosome. Stable ubiquitous overexpression of DmHSP23 and DmHSP27 was confirmed by Westernblotting with specific antibodies. Other *Drosophila* small HSP overexpressing strains included were HSP67Bc, CG4461, CG7409, CG14207.<sup>22,23</sup> These transgenic strains were generated at Genetic Services Inc. by injection of the pUAS vector containing a small HSP of interest with a V5 tag, into the W1118 genetic background. These transgenic strains were crossed with actin-GAL4 expressing flies in order to induce ubiquitous transgenic gene expression of the small DmHSP of interest in the F1 progeny, which was confirmed by Westernblotting.

Heat treatment was performed at 37 °C for 1 hour followed by an overnight recovery at 25 °C. The HSP-inducing compounds Geranylgeranylacetone (GGA, 1mM) and O-(3-piperidino-2-hydroxy-1-propyl)-nicotinic acid amidoxim di-hydrochloride (BGP-15, 1mM) (both kind gifts from Eisai Japan and NP-gene USA, respectively) were freshly dissolved in de-mineralized water and 0.5 ml was added to standard *Drosophila* food for at least 48 hours before (tachy)pacing. Controls were subjected to de-mineralized water only.

### 2.2. Tachypacing of *Drosophila* and measurement of heart function parameters

Early pupae were selected as described before.<sup>24</sup> In short, transparant pupae were selected at entry of the immobile phase (this phase continues for about 3 hours) and were placed on 1% agarose gel in PBS. The basal heart rate in the early pupae was about 2.2Hz. Pupae were tachypaced by using the C-Pace100™-Culture Pacer (IonOptix Corporation, The Netherlands) for 0-120 minutes. Controls were subjected to normal electrical field stimulation similar to basal heart rate (2.2Hz) whereas tachypacing was conducted at 2.3 fold basal rate or as indicated. Under the light microscope, heart function at each time point was visually scored in duplicate each for a period of 20 seconds. In short, the rate of contraction of the heart was quantified by focussing on the heart wall contrast edges.<sup>24</sup> During (tachy)pacing, time lapse movies were made and analysed by edge detection software (Leica Microsystems, Mannheim, Germany) to determine the heart wall shortening, which indicates

the strength of contraction (amplitude of diastolic and systolic heart wall contraction), and duration of arrhythmic periods (ImageJ).<sup>24</sup> Arrhythmicity index was calculated as the duration of arrhythmic periods divided by the total duration of measured periods.

### 2.3. Quantitative PCR

From at least five early pupae per condition, total RNA was isolated by utilizing the Nucleospin RNA/protein mini kit (Macherey-Nagel, The Netherlands). First strand cDNA was generated using M-MLV reverse transcriptase (Invitrogen, The Netherlands) using oligo(dT)18 primers (Biologio, The Netherlands). Relative changes in transcription level were determined using the CFX384 Real-Time System C1000 Thermocycler (BioRad, The Netherlands) in combination with SYBR green supermix (Bio-Rad, The Netherlands). Calculations were performed using the comparative CT method according to User Bulletin 2 (Applied Biosystems). Fold induction was adjusted using RpL32 transcript levels as a standard. Primer pairs used included the heat inducible DmHSP27 F: CTAGACAGGGTTGTGAATAAAGAG and R: AAACCGAAGTCATCCTCCAG and DmHSP70AA F: ACCTCAACCTATCCATCAACC and R: GTCTCAATTCCCAATGAAAGTG and for RpL32 F: CGATCTCGCCGCAGTAAA and R: GCACCAAGCACTTCATCC. The PCR efficiencies for all primer pairs were between 85% and 100%.

### 2.4. Western-blot analysis

*Drosophila* pupae or adult flies (five per condition) were quickly lysed on ice in 50 µl SDS sample loading buffer (10% SDS, 50% glycerol, 0.33M Tris-HCl pH 6.8, 10% β-mercaptoethanol, 0.05% bromophenol blue) followed by sonification. After a short spin-down, supernatant was collected and boiled for 6 minutes. Proteins were separated on SDS-PAGE 4-20% Precise™ Protein gels (Thermo Scientific) and transferred onto nitrocellulose membranes (GE Healthcare, The Netherlands). The membranes were blocked in 5% skim milk (1 hour, room temperature) and incubated overnight at 4°C with primary *Drosophila* anti-DmHSP23, anti-DmHSP27 antibodies<sup>25,26</sup>, anti-V5 antibodies (Invitrogen, The Netherlands) or anti α-tubulin antibodies (Sigma, The Netherlands). Horseradish peroxidase-conjugated anti-mouse (Santa-Cruz Biotechnology, The Netherlands) was used as secondary antibody. Signals were detected by Super Signal (Thermo Scientific, The Netherlands) and quantified by densitometry.

### 2.5. (Ultra)structural evaluation by Light and Electron Microscopy

For morphological evaluation, the head and the abdomen were dissected from the early pupae and the middle segment was immediately fixed for at least 2 hours at 4°C in 2%

glutaraldehyde (in 0.1M cacodylate buffer, pH7.4). Post-fixation was performed for 2 hours in 1% osmium tetroxide (supplemented with 1.5%  $K_4Fe(CN)_6$  in cacodylate buffer, pH7.4) at 4°C. After dehydration in ethanol, pupae were embedded in Epon and semi-thin sections (1  $\mu$ m) were cut and stained with 1% toluidine blue and used for light microscopic evaluation. To verify the ultrastructural changes, ultrathin sections (60nm) were made and stained with uranylacetate and lead citrate and examined in a Philips CM100 electron microscope operating at 60kV. Changes were evaluated in six randomly chosen regions by an investigator blinded for *Drosophila* groups, who (1) calculated the ratio of area positive for myolysis (areas with >10% loss of sarcomeres) compared to total area, using ImagePro software<sup>27</sup>; and (2) scored mitochondrial degeneration by determining the ratio degenerated versus total mitochondria.

## **2.6. Statistical analysis**

Results are expressed as mean $\pm$  SEM. All experimental procedures were performed in at least duplicate series. ANOVA was used for multiple-group comparisons. Student *t* tests were used for comparisons involving only 2 groups, and *t* tests with Bonferroni correction were used to compare individual group differences when multiple-comparison ANOVA was significant. All *P* values were two-sided. *P*<0.05 was considered statistically significant. SPSS version 16.0 was used for statistical evaluation.

## **3. RESULTS**

### **3.1. Tachypacing of *Drosophila* induces contractile dysfunction in the heart wall**

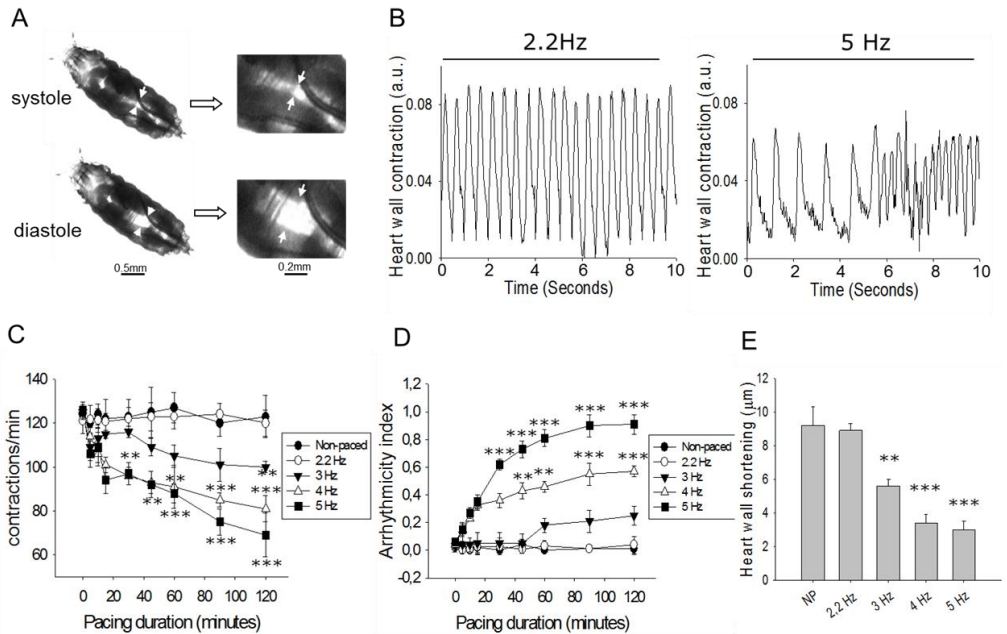
Early *Drosophila* pupae possessed a basal rate of 2.2Hz  $\pm$  0.2Hz and were subjected to tachypacing at 3, 4 and 5 Hz for up to 2 hours, and contractile function of the heart wall was determined. It was found that tachypacing results in a gradual and significant reduction in heart contraction rate (Figure 1A, B). In addition, tachypacing also induced a significant induction in arrhythmic episodes (Figure 1A, C). Moreover, increasing frequency of tachypacing induced a gradual reduction in the amplitude of diastolic and systolic heart wall shortening (Figure 1D). Since tachypacing at 5Hz, a 2.3 fold rate increase compared to basal heart rate, significantly induced contractile dysfunction of the heart wall, this setting was used in all following tachypacing experiments.

### **3.2. Heat shock and the HSP-inducers GGA and BGP-15 increase HSP levels in *Drosophila* and protect against tachypacing-induced contractile dysfunction**

In *in vitro* cardiomyocyte and *in vivo* canine models for AF, it was observed that HSP-inducing treatments protect against tachycardia remodeling.<sup>3,7</sup> In order to determine if the

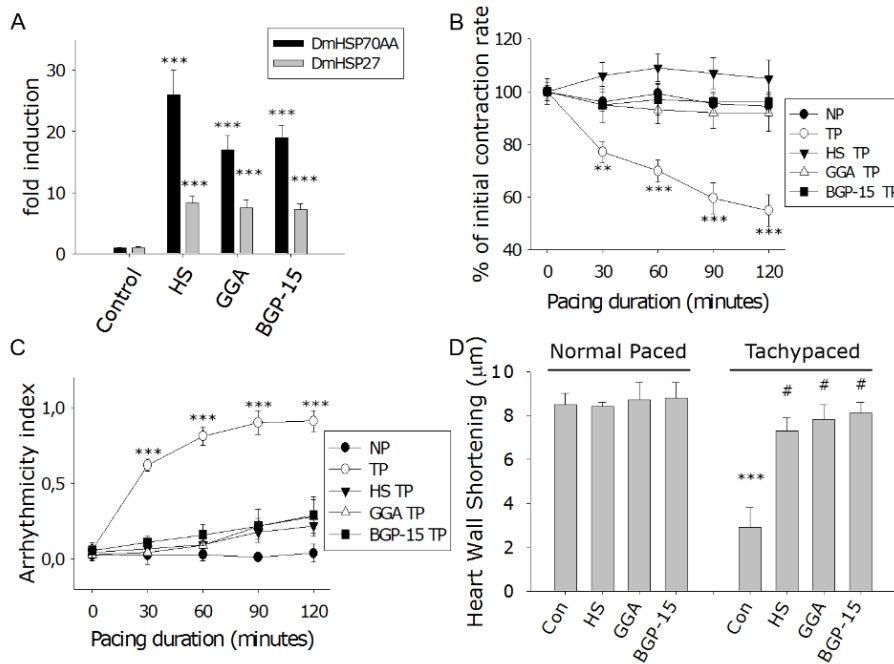
same result can be obtained in tachypaced *Drosophila*, fly stocks were subjected to a heat treatment (1 hour 37°C, 16 hours recovery), or pretreatment with the HSP inducing agents GGA or BGP15. Quantitative polymerase chain reaction with reverse transcription (RT-PCR) was used to determine the relative abundance of endogenous DmHSPs. All three HSP-inducing treatments effectively induced the heat shock response as evidenced by an increase in mRNA levels of endogenous DmHSP70AA and DmHSP27 (Figure 2A).

All HSP inducing treatments protected against tachypacing-induced reduction in heart contraction rate (Figure 2B), increase in arrhythmic episodes (Figure 2C) and reduction in amplitude of heart wall shortening (Figure 2D). These results indicate that boosting the heat shock response results in protection against tachypacing-induced contractile dysfunction of the heart in *Drosophila* pupae, thus further validating *Drosophila* as an *in vivo* model for tachycardia remodeling.



**Figure 1: Tachypacing of *Drosophila* induces heart wall remodeling, including gradual reduction in contractile function, induction in arrhythmic periods and reduction in heart wall shortening.** A) Illustrations of heart wall contractions are depicted after 2 hours normal pacing (2.2Hz) and tachypacing (5Hz) of early pupae of a W1118 genetic background. B) Rate of heart wall contractions are depicted for non-paced, normal paced (2.2Hz) and tachypaced (3,4 and 5Hz) early pupae. C) Arrhythmicity index of non-paced, normal paced (2.2Hz) or tachypaced (3,4 and 5Hz, 2 hours) wild-type early pupae. D) Amplitude of heart wall shortening of normal paced (2.2Hz) or tachypaced (3,4 and 5Hz, 2 hours) wild-type early pupae. N=13-20 early pupae per condition. \*\* $P < 0.01$  vs Control normal paced, \*\*\* $P < 0.001$  vs Control normal paced.

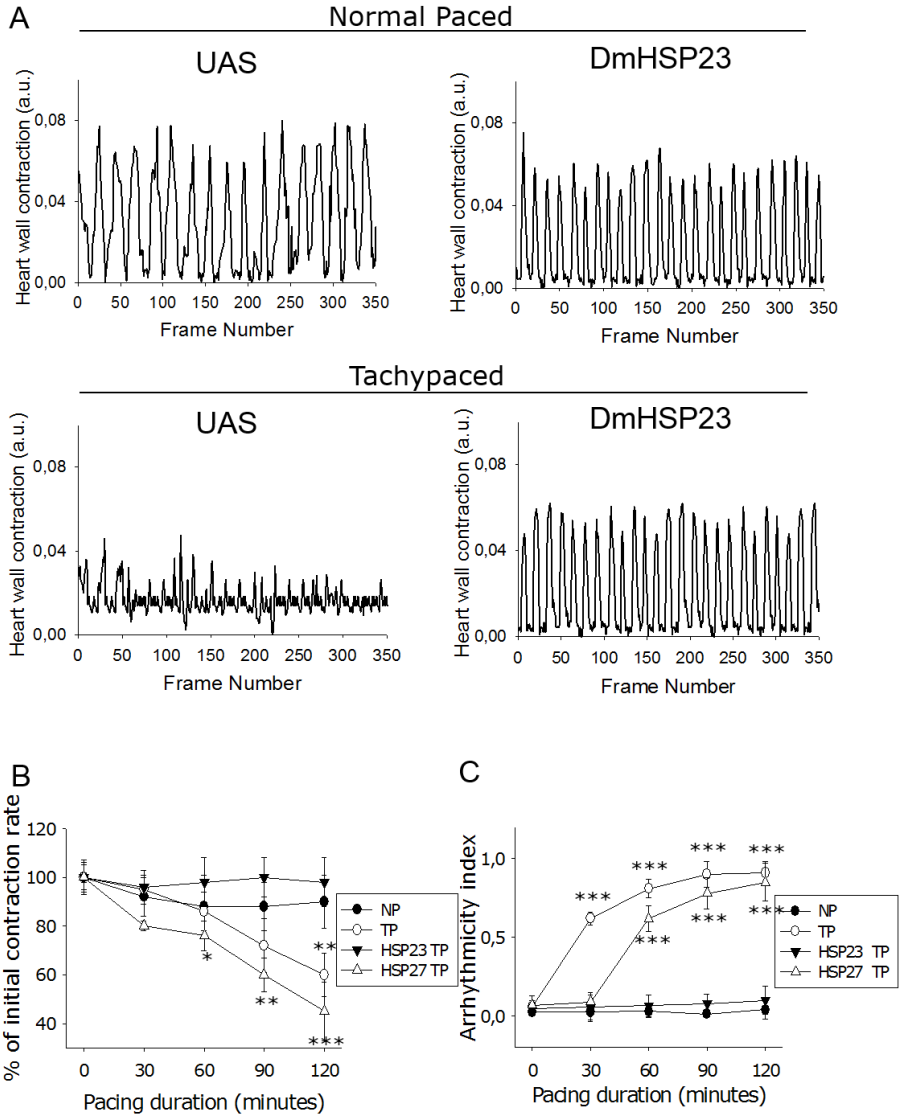




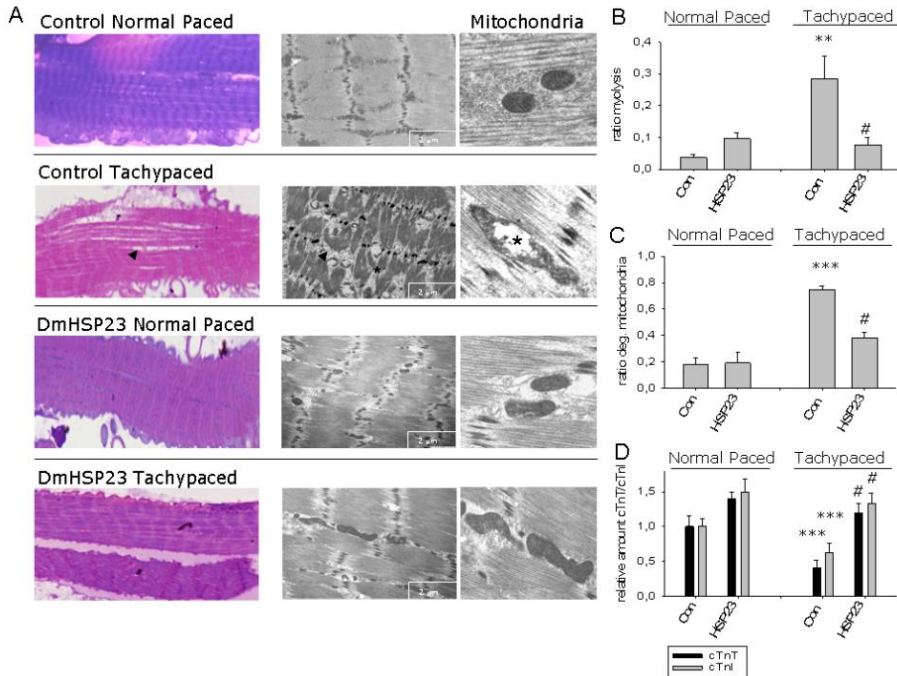
**Figure 2: HSP-inducing treatments protect against tachycardia remodeling in *Drosophila*.** A) A mild heat shock (HS), GGA or BGP-15 pretreatment induces the amount of mRNA of *Drosophila* DmHSP27 and DmHSP70AA compared to RpL32 levels as a standard, as determined by QPCR. B) The percentage of initial contraction rate is depicted for normal paced (NP) and tachypaced (TP) early W1118 pupae with or without HS, GGA or BGP-15 pretreatment. C) Arrhythmicity index of normal paced (NP) and tachypaced (TP) early pupae with or without HS, GGA or BGP-15 pretreatment. D) Degree of heart wall shortening of normal paced or tachypaced (2 hours) wild-type early pupae with or without HS, GGA or BGP-15 pretreatment. N=15-20 early pupae per condition. \*\* $P < 0.01$  vs Control, \*\*\* $P < 0.001$  vs Control, # $P < 0.01$  vs Control tachypaced.

### 3.3. *DmHSP23* overexpression protects against tachypacing-induced contractile dysfunction and structural changes of the heart wall.

Previously, we observed a protective effect of HSPB1, the most prominent heat inducible cytosolic member of the human family of small HSPs, against tachycardia remodeling in HL-1 atrial cardiomyocytes.<sup>3</sup> To further validate our model, it was tested if the cytosolic heat inducible member of the *Drosophila* family of small HSPs, DmHSP23<sup>19,28</sup>, protects against tachycardia remodeling. Indeed, the DmHSP23 overexpressing transgenic strain (Table 1) was protected against tachypacing-induced reduction in heart contraction rate (Figure 3A, B), increase in duration of arrhythmic episodes (Figure 3C) and also reductions in amplitude of heart wall shortening (Figure 5C). In addition, (ultra)structural evaluation of the heart wall indicates that tachypacing induces loss of sarcomeres (myolysis, Figure 4A, B) and degeneration of the mitochondria (Figure 4A, C), including occasional enlargement and disorganization of cristae (Figure 4A). All these (ultra)structural changes were significantly attenuated in tachypaced DmHSP23 overexpressing *Drosophila* strain (Figure 4B, C).



**Figure 3: DmHSP23 but not DmHSP27 overexpressing *Drosophila* are protected against tachycardia remodeling.** A) Heart wall contractions are depicted for normal paced (NP) and tachypaced (TP, 2 hours) early pupae with UAS-DmHSP23 genetic background (no DmHSP23 overexpression, UAS) and with a UAS-actinGAL4-DmHSP23 (DmHSP23) overexpressing background. B) The percentage of initial contraction rate is depicted of normal paced (NP) and tachypaced (TP) early pupae of control UAS, DmHSP23 and DmHSP27 overexpressing strains. C) Arrhythmicity index of normal paced (NP) and tachypaced (TP) early pupae of control UAS, DmHSP23 and DmHSP27 overexpressing strains. N=12-20 early pupae per condition. \* $P < 0.05$ , \*\* $P < 0.01$  vs Control, \*\*\* $P < 0.001$  vs Control.

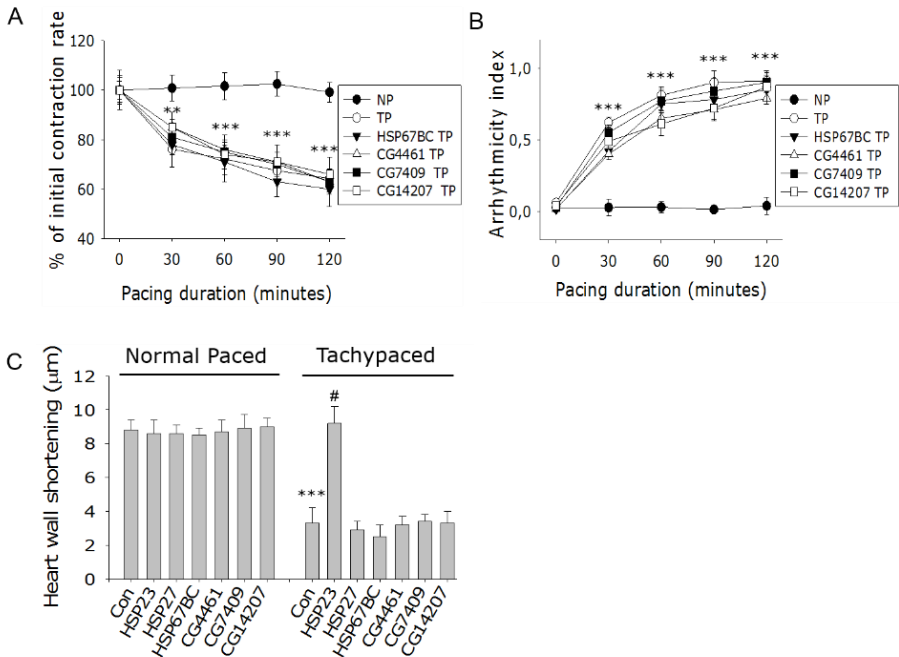


**Figure 4: DmHSP23 overexpressing *Drosophilas* are protected against tachypacing-induced structural remodeling, including loss of sarcomeres (myolysis) and mitochondrial damage.** A) Left panel shows light microscopic structure of the heart wall in normal paced and tachypaced control (UAS-DmHSP23) and DmHSP23 (UAS-actinGAL4-DmHSP23) overexpressing strains. Right panel shows electron microscopic details of the cardiomyocytes and mitochondria. Arrowheads show loss of sarcomeres in the heart wall and asterisk marks mitochondrial damage. B) Quantification of ratio myolysis compared to total area of sarcomeres and C) ratio degenerated mitochondria compared to total amount of mitochondria in normal paced and tachypaced control (UAS-DmHSP23) and DmHSP23 (UAS-actinGAL4-DmHSP23) overexpressing strains. N=3-5 early pupae per condition \*\* $P < 0.01$  vs Control normal paced, # $P < 0.05$  vs Control tachypaced.

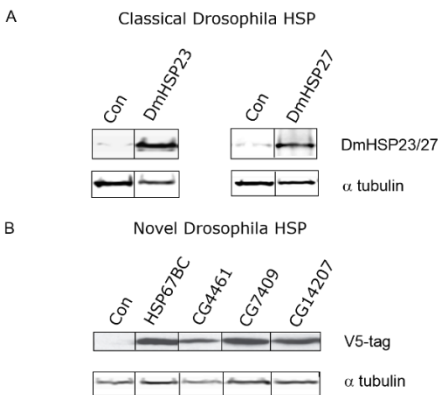
### 3.4. Unravelling the mechanism of DmHSP23 protection against tachycardia remodeling, by comparing small HSP overexpressing *Drosophila* strains.

Having established that tachypacing of *Drosophila* results in contractile dysfunction, which can be prevented by general overexpression of HSPs and in particular DmHSP23, we next used a genetic approach to get more mechanistic insight in its mode of protection. Hereto, we used five *Drosophila* strains overexpressing different small HSPs with various cellular localization and functions in protein refolding, protein aggregation and autophagy (Table 1). Overexpression of DmCG4461, which lacks these functions, was also ineffective in protection against tachycardia remodeling (Figure 5). Next, it was tested whether the protective effect of DmHSP23 requires its cytosolic localization. Hereto, we used flies overexpressing DmHSP27, which is exclusively localized in the nucleus but otherwise shares most (functional) features of DmHSP23.<sup>26,28-31</sup> As can be seen in Figure 3B,C DmHSP27 did not result in protection against AF, indicating that a protein refolding activity in the nucleus

is insufficient for the protective effect. Yet, overexpression of the DmCG7409, a cytosolic protein that has a strong activity to support protein refolding, did also not reveal tachycardia protection, suggesting that the remodeling induced by tachycardia cannot be prevented by the refolding-like activities of DmCG7409. Overexpression of DmHSP67Bc, which lacks this classical chaperone-like activity (Table 1)<sup>23</sup> but which induces autophagy and hereby assists in the clearance of toxic protein aggregates<sup>22</sup>, also did not protect against tachycardia remodeling (Figure 5). In addition, overexpression of (the non-heat inducible) DmCG14207, which enhances protein refolding<sup>23</sup> and associates with Z bands in muscles<sup>32</sup>, did not protect against tachycardia remodeling (Figure 5). Overexpression of the small HSP of interest was confirmed by Western blot analysis (Figure 6). Thus, our findings suggest that the effectiveness of DmHSP23 to protect against tachycardia remodeling is due to a specific cytosolic feature related to tachycardia, which is unrelated to protein refolding or autophagic capacities, and can not be prevented or repaired by all other small HSP members.



**Figure 5: HSP67Bc, CG4461, CG7409 and CG14207 show no protective effects against tachycardia remodeling in *Drosophila*.** A) The percentage of initial contraction rate is depicted for normal paced (NP) and tachypaced (TP) early pupae of control W1118-actinGAL4 and UAS-actinGal4-HSP67Bc, -CG4461, -CG7409 or -CG14207 overexpressing strains. B) Arrhythmicity index of normal paced (NP) and tachypaced (TP) early pupae of control and HSP67Bc, CG4461, CG7409 or CG14207 overexpressing strains. C) Combined results of degree of heart wall shortening of normal paced or tachypaced (2 hours) early pupae of control and DmHSP23, DmHSP27, HSP67Bc, CG4461, CG7409 or CG14207 overexpressing strains. N=11-15 early pupae per condition. \*\*\* $P < 0.001$  vs Control NP, # $P < 0.001$  vs Control TP.



**Figure 6: Western blot showing overexpression of the individual small DmHSPs in transgenic *Drosophila* strains.** A) Representative blot showing overexpression of the classical *Drosophila* small HSPs, DmHSP23 (UAS-actinGAL4-DmHSP23) and DmHSP27 (UAS-actinGAL4-DmHSP27), compared to respectively control UAS-DmHSP23 or UAS-DmHSP27 genetic background. B) Representative blot showing overexpression of novel *Drosophila* small HSPs, UAS-actinGAL4-HSP67Bc, -CG4461, -CG7409 or -CG14207, compared to control actinGAL4-W1118 genetic background. Alpha-tubulin was used as a loading control.

**Table 1: Overview of *Drosophila* transgenic small HSP overexpressing lines used in the current study.**

<i>Drosophila</i> small HSP	Name	Function	Reference
Classical HSPs	HSP23	- heat inducible	25, 30
		- assists protein refolding	
	HSP27	- heat inducible	25,28,30
		- assists protein refolding	
Novel HSPs	HSP67Bc	- heat inducible	24,25
		- prevents protein aggregation	
		- induces autophagy	
	CG4461	- heat inducible	25
	CG7409	- mild heat inducible	25
		- assists protein refolding	
	CG14207	- not heat inducible	25
		- assists protein refolding	

4. DISCUSSION

Previously, we reported on cardioprotective effects of HSPs, especially the small human HSPB1, and the HSP-inducing agent GGA against important features of tachycardia remodeling, such as electrical and structural remodeling and contractile dysfunction.<sup>3,5-7</sup> In the present study, we demonstrated for the first time that the protective effects are also observed *in vivo* in the *Drosophila melanogaster* model for tachypacing-induced contractile dysfunction of the heart wall. We observed that both HSP-inducing agents GGA and BGP-15 but also a heat shock pretreatment protect against tachypacing-induced contractile dysfunction. By comparing in transgenic *Drosophila* strains the overexpression of six

individual small HSPs, it was found that only DmHSP23 resulted in cardioprotective effects. Moreover, our findings indicate that similar findings observed in *in vitro* tachypaced HL-1 and dog atrial cardiomyocytes and *in vivo* canine model for AF, *Drosophila melanogaster* can be used to study tachycardia remodeling. Since *Drosophila* can be easily utilized to manipulate gene expression in a highly precise spatial and temporal fashion, this model of AF thus seems to represent an excellent tool for studying molecular mechanisms of heat shock-mediated cardioprotection and genes involved in tachycardia remodeling and AF progression.

#### **4.1. Tachypaced *Drosophila melanogaster* as model system for contractile dysfunction of the heart wall**

The *Drosophila* heart is a simple tube that runs along the dorsal midline of the pupae.<sup>33</sup> The heart is composed of two major cell types: cardioblasts, which form the heart tube and are the contractile cells of the heart, and pericardial cells, which flank the cardioblasts but do not express muscle proteins.<sup>33</sup> Moreover, *Drosophila* myocardium contains myogenic cardiac pacemaker cells, which respond to a variety of neurotransmitters and hormones by adjusting heart rate.<sup>34</sup> Tachypacing of the *Drosophila* hearts induced cardiomyocyte remodeling that was comparable to the remodeling observed in *in vitro* tachypaced cardiomyocytes<sup>4,35</sup>, *in vivo* animal models for AF<sup>36,37</sup> and clinical AF.<sup>38,39</sup> Moreover, the cardioprotective effect of the general induction of the heat shock response, that was previously shown to be protective in *in vitro*<sup>3,5</sup> and *in vivo*<sup>3,7</sup> AF models, was also found to be protective in the tachypaced *Drosophila* model. Finally, also overexpression of a single small HSP, DmHSP23, revealed the same cardioprotective effects as seen for human HSPB1 in tachypaced HL-1 cardiomyocytes. Together, the data validate the tachypaced *Drosophila* model as versatile model system for tachycardia remodeling and AF progression.

#### **4.2. Small heat shock proteins in *Drosophila melanogaster***

The family of *Drosophila melanogaster* small HSPs comprises 12 proteins, but only four of them have been studied in greater detail and form the classical DmHSPs, DmHSP22, DmHSP23, DmHSP26 and DmHSP27.<sup>23</sup> Although these four proteins share high homology, their intracellular localization differs.<sup>23,28</sup> DmHSP22 is localized to mitochondria<sup>40</sup>, DmHSP23 and DmHSP26 were found in the cytosol<sup>28,29</sup> and DmHSP27 is present in the nucleus.<sup>30</sup> It is unknown which member of the *Drosophila* small HSP family represents the functional ortholog of human HSPB1. Based on the heat inducibility of HSPB1, its cytoplasmic localization, and its ability to function in the HSPA1A dependent refolding activity, DmHSP23 and DmHSP26 most closely resemble HSPB1.<sup>23</sup> DmHSP26 is, however, rather effective in protection against aggregation of polyglutamine proteins that are

associated with neurodegenerative disease<sup>28</sup>, a property that is lacking for DmHSP23<sup>28</sup> and human HSPB1.<sup>41</sup> Although we did not include flies overexpressing DmHSP26, we now show that DmHSP23, like HSPB1, also protects against tachycardia remodeling. This finding suggests that DmHSP23 might represent a functional ortholog of human HSPB1.

The question arised which small HSP activity is required for cardioprotection in AF. In a recently completed study comparing all 10 human HSPB members in a HL-1 cardiomyocyte model for AF<sup>42</sup>, we found three members, HSPB1, HSPB6, and HSPB7, to strongly protect against tachycardia remodeling and one member, HSPB8, had minor protective actions. DmHSP67Bc was recently found to be the functional ortholog of the human HSPB8<sup>22</sup>, and was ineffective in the current study. This implies that the autophagy stimulating function of the human HSPB8<sup>22,43,44</sup> and DmHSP67Bc<sup>22</sup> is insufficient to protect against tachycardia remodeling in *Drosophila*. The function shared by HSPB1, HSPB6, and HSPB7 that was found responsible for cardioprotection, was their ability to bind to actin and to prevent actin polymerization<sup>42</sup> and not their association with canonical activities of HSPBs like refolding of denatured proteins.<sup>42</sup> Also in the current study, two cytosolic members of the *Drosophila* small HSP family that do function in refolding, CG7409 and CG14207, do not protect against tachycardia remodeling. This suggests that neither CG7409 nor CG14207 are likely to be functional orthologs of HSPB1, HSPB6, or HSPB7. The same is true for CG4461, for which no clear activity has been found so far. Finally, flies overexpressing DmHSP27, which is exclusively localized in the nucleus but otherwise shares most (functional) features of DmHSP23<sup>28</sup> did not result in protection against tachycardia remodeling, indicating that cytoplasmic activity is required for a protective effect. Based on the studies with the human HSPB members<sup>42</sup>, actin binding may be a key feature required for such cardioprotective activity. Whether DmHSP23 indeed can bind actin has not been demonstrated so far. Only for HSP67Bc and CG14207, it was shown that they associate with Z bands in muscles<sup>22,32</sup>, but neither one of these two members protected against tachycardia remodeling in *Drosophila*, indicating that this feature is not associated with the observed cardioprotective effect. Furthermore, it might be argued that the V5 tag of the novel small HSPs is suppressing its function, but the HSP67Bc, CG14207 and CG7409 transgenic strains were found to be efficient in preventing aggregation of disease related misfolded proteins, indicating no functional interference of the V5 tag.<sup>23</sup> Thus, the precise mechanism for the effectiveness of DmHSP23 to protect against tachycardia remodeling remains to be determined, but there are indications that a specific tachycardia-related feature of DmHSP23 that is not shared by the other five small DmHSP members, plays a key role. The combined data also demonstrate that, next to sharing common functions in proteostasis, small HSP seem to have distinct client specific functions.

In summary, the tachypaced *Drosophila* model for AF as shown in the current study, provides a versatile system to study both mechanism of tachycardia remodeling and disease

progression, to search for genetic modifiers of tachycardia remodeling, and to screen for pharmacological compounds that can prevent AF mediated cardiac damage.

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